BMA BIOMEDICALS

Peninsula Laboratories

BMA Biomedicals

Peptide ELISA Protocol for S-1502

EIAH - high sensitivity absorbance assays

Note: Investigational Device Limited by Law to Investigational Use
For Research Use Only, not for Use in Diagnostic Procedures

Technical Help and Ordering Information:
Please e-mail your questions or requests to info@bma.ch

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STORAGE

After you receive the kit, store the lyophilized components at a constant -20°C for up to one year from the kit's assembly date. The remaining components should be stored in the refrigerator (4-8°C) also up to 1 year. Long term storage, improper storage conditions and large temperature fluctuation cycles may result in the accumulation of precipitates in the TMB solution and in the ELISA buffer concentrate. These precipitates should not affect the assay noticeably. Nevertheless, if you observe such precipitates, avoid them by allowing them to sink to the bottom.

KIT COMPONENTS

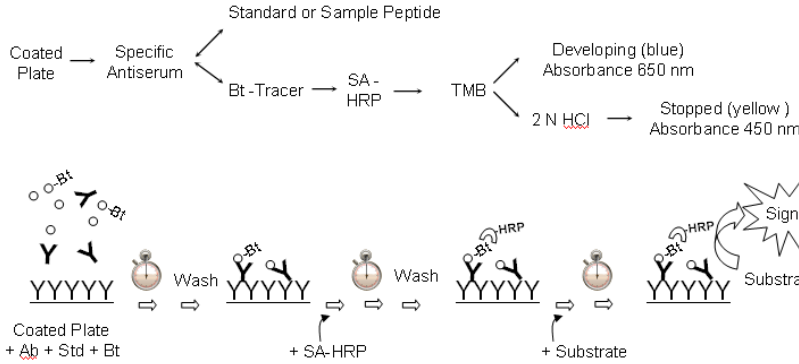
Materials Provided (Please consult “Prepare Kit Components” on page 5)

- EIA Buffer Concentrate (50 ml 20x concentrate)
- 96-well immunoplate with acetate plate sealer
- Antiserum (lyophilized powder)
- Biotinylated tracer (lyophilized powder)
- Streptavidin-HRP (100µl 200x concentrate)
- TMB substrate solution (1.5ml)
- TMB substrate buffer (15ml citrate buffer)
- Stop Solution 2N HCl (15ml)
- Kit Datasheet/Protocol Insert

Materials Not Provided

- Standard Peptide. Purchase synthetic peptide with sequence identical to the antigen sequence listed on the datasheet.
- 96-well microtiter plate reader set up to measure 450nm and 650nm.
- 96-well plate washer and shaker **(optional)**
- Distilled, deionized or USP water
- Curve fitting software (**optional**)
- Test tubes, pipettes and various other standard laboratory items
- Extraction Kit (with 50 SEP columns and Buffers A and B, Catalog# S-5000) (**recommended**)
    - Buffer A (Catalog# Y-1040)
    - Buffer B (Catalog# Y-1045)
    - SEP columns (200 mg) (Catalog# Y-1000)
    - SEP column adapters (Catalog# Y-1010)

BASIC NOTIONS AND FACTS



This ELISA kit is a competitive immunoassay. The **antiserum** is captured by antibodies coated on a 96-well plate. A constant concentration of **Bt-tracer** (biotinylated tracer) and varying concentrations of unlabeled standard or sample peptide compete for binding specifically to the antiserum. Captured Bt-tracer is subsequently bound by **SA-HRP** (streptavidin-conjugated horseradish peroxidase), which produces a soluble colored product after a **substrate** is added.

The **sequence of the antigen** is shown on page 1, under Immunogen.

Make a **standard curve** in the **range** specified in the kit's **datasheet**. Standard curves are **S-shaped** (on a **semi-log plot**) or almost linear over the kit's range. The **measuring range** is the range of standard concentrations near the middle or near the **IC_{50}** of the standard curve. Unknown sample concentrations are measured by comparing their absorbance with the standard curve.

We include sufficient reagents for **96 determinations.**

Variation • Accuracy • Extraction • Cross-reactivity
The kit's IC_{50}, or the shape of the standard curve, may exhibit some variation but this will not affect the kit's accuracy in the measuring range. The kit accurately measures sample peptides if the following conditions are met.

A) Both samples and standards must be measured in the same diluent and under the same conditions (same microtiter plate).

B) The kit's antiserum must not cross-react appreciably with other factors present in the sample. Cross-reactivity tables are included with each kit. The user may wish to test the cross-reactivity with other peptides.

C) The sample peptides must be identical to the standard. Ideally the synthetic standard mimics the natural peptide perfectly. Sometimes, however, natural peptides exist as families of species related by a common or similar sequence. In addition, natural peptides may be enzymatically or spontaneously modified, may exist in complexes, and may assume alternative structural forms. In these cases the kit might not measure the exact concentration of a particular natural peptide species, but, it may still be used for relative average measurements.

D) Sample extraction. Factors present in serum can bind to kit components. The effects can vary from negligible to complete obliteration of signal. Sample extraction may, therefore, be required prior to using the kit.

PROTOCOL NOTES AND ASSAY PREPARATION

EIA buffer. The antiserum and the Bt-tracer are reconstituted and used in ELISA buffer. The standard dilutions and samples are prepared in ELISA buffer.
Room Temperature. Reagents, samples, and the plate should be brought to room temperature before use.
Shakers. Shakers (optional) may help lower the experimental variation of duplicates (recommended at 60 rpm).
Blank Wells. Blanks will give you the background to be subtracted from all readings. These should not be confused with the "S0 Standards" which contain no standard peptide and which will yield the highest readings. Blank readings will not influence concentration calculations - thus, they are optional.

PLATE LAYOUT: Seven-Point Standard Curve

	1	2	3	4	5	6	7	8	9	10	11	12
A	**Blk**	**Blk**	**U1**	**U1**	**U9**	**U9**	**U17**	**U17**	**U25**	**U25**	**U33**	**U33**
B	**S1**	**S1**	**U2**	**U2**	**U10**	**U10**	**U18**	**U18**	**U26**	**U26**	**U34**	**U34**
C	**S2**	**S2**	**U3**	**U3**	**U11**	**U11**	**U19**	**U19**	**U27**	**U27**	**U35**	**U35**
D	**S3**	**S3**	**U4**	**U4**	**U12**	**U12**	**U20**	**U20**	**U28**	**U28**	**U36**	**U36**
E	**S4**	**S4**	**U5**	**U5**	**U13**	**U13**	**U21**	**U21**	**U29**	**U29**	**U37**	**U37**
F	**S5**	**S5**	**U6**	**U6**	**U14**	**U14**	**U22**	**U22**	**U30**	**U30**	**U38**	**U38**
G	**S6**	**S6**	**U7**	**U7**	**U15**	**U15**	**U23**	**U23**	**U31**	**U31**	**U39**	**U39**
H	**S0**	**S0**	**U8**	**U8**	**U16**	**U16**	**U24**	**U24**	**U32**	**U32**	**U40**	**U40**

Blk = blank S = standards U = unknown samples

PREPARE SAMPLES AND STANDARD DILUTIONS

1. Sample extraction. Sample extraction is recommended especially for serum samples. It may not be as important for some tissue culture samples. See "Suggested Protocol for Sample Extraction" below for details. The kit may still be used without extraction but this may cause unexpected results due to the possible binding between serum proteins and kit components.

2. Sample concentration. The concentration of the target molecule must be within the measuring range of the kit (in a region around the IC_{50}). If you cannot estimate the concentration range of your sample you can prepare it at different concentrations such that one of the samples may be within the measuring range.

3. Standard. Purchase synthetic peptide corresponding to the antigen sequence. Make a stock solution from which to prepare the serial dilutions for the standard curve. Make serial dilutions of the purchased standard to cover the range of the kit, starting at 100ng/ml ("S1"), making 4-fold serial dilutions through S6**.**

PREPARE KIT COMPONENTS

Lyophilized kit components should not be re-hydrated until they are needed. **Please read the complete protocol before proceeding.**

1. Equilibrate unopened kit components to room temperature. To avoid accumulation of moisture, do not open reagents and immunoplate while they are cold.

2. ELISA buffer: Dilute the ELISA buffer concentrate to 1000ml with distilled, deionized or USP pure water and mix well.

3. Antiserum: Add 5ml ELISA buffer and vortex gently.

4. Biotinylated tracer: Add 5ml ELISA buffer to the vial of lyophilized biotinylated peptide and vortex gently.

5. TMB chromogenic solution (Step 9 of the following protocol): Prepare TMB chromogenic solution immediately before use by mixing 20 parts of the TMB substrate buffer (citrate, brought to room temperature) with 1 part TMB - H_2O_2 solution (TMB substrate stock). ***Example:*** mix 0.5ml TMB-H_2O_2 substrate stock into 10ml substrate buffer. This dilution should be used within 15 minutes after preparation

PROTOCOL

1 - Into each well of the immunoplate, except blank wells, add

50µl standard or sample (in ELISA buffer)

25µl antiserum (in ELISA buffer)

Add 75µl ELISA buffer to blank wells.

2 - Incubate at room temperature for 1 hour. Shorter pre-incubations may result in lower sensitivity.

3 - Rehydrate the Bt-tracer (in ELISA buffer) **and add 25µl per well.**

4 - Incubate at room temperature for 2 hours.

5 - Wash immunoplate 5 times with 300µl per well of ELISA buffer. Be very careful not to cross-contaminate between wells in the first wash/dispensing cycle. In each wash cycle, empty plate contents with a rapid flicking motion of the wrist, then gently blot dry the top of plate on paper towels. Dispense 300µl of ELISA buffer into each well and gently shake for at least a few seconds. Thorough washing is essential.

6 - Add 100µl per well of streptavidin-HRP. Tap or centrifuge the SA-HRP vial to collect all liquid contents on the bottom of the vial. Dilute 1/200 in ELISA buffer (60µl in 12ml) and vortex gently. Add 100µl to all wells, including the blanks.

7 - Incubate at room temperature for 1 hour.

8 - Wash immunoplate 5 times (see step 5).

9 - Add 100µl per well of TMB chromogenic solution. (A mixture of 1 part TMB-H2O2 stock in 20 parts citrate buffer). Add to all wells, including the blanks. Use an eight channel pipette to obtain even results.

10 - Incubate at room temperature (usually 5 - 15 minutes). You may read the developing blue color at 650nm and use the data for your calculations.

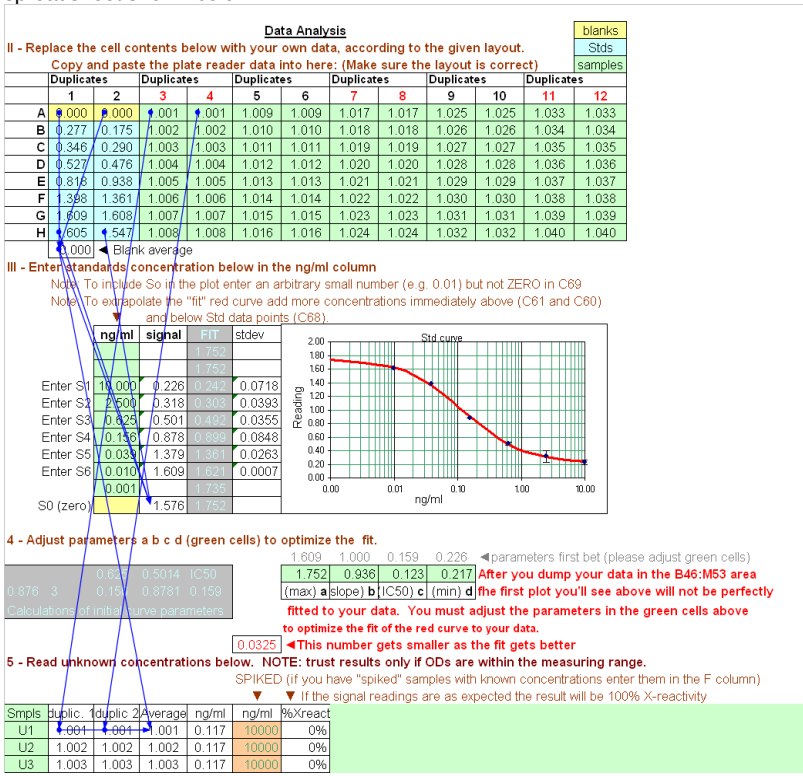
11 - Terminate reactions by adding 100µl 2N HCl per well.

12 - Read absorbance at 450nm within fifteen minutes.

DATA ANALYSIS

Plot data and calculate results. We recommend that you use curve fitting software for your data analysis. Plate readers often include such software packages. This is, however, not essential and you may opt to plot manually on semi-log paper. You can also use a spreadsheet program. Should you need help with the latter method we recommend the following procedure.

Set up a spreadsheet as shown below (note that the values on the spreadsheet are merely illustrative and are not necessarily typical for this particular kit). If you e-mail us (contact information on front cover) we will be happy to send you the actual working Excel spreadsheet shown below.



Data Analysis

blanks
Stds
samples

II - Replace the cell contents below with your own data, according to the given layout.
Copy and paste the plate reader data into here: (Make sure the layout is correct)

	Duplicates		Duplicates		Duplicates		Duplicates		Duplicates		Duplicates	
	1	2	3	4	5	6	7	8	9	10	11	12
A	0.000	0.000	1.001	1.001	1.009	1.009	1.017	1.017	1.025	1.025	1.033	1.033
B	0.277	0.175	1.002	1.002	1.010	1.010	1.018	1.018	1.026	1.026	1.034	1.034
C	0.346	0.290	1.003	1.003	1.011	1.011	1.019	1.019	1.027	1.027	1.035	1.035
D	0.527	0.476	1.004	1.004	1.012	1.012	1.020	1.020	1.028	1.028	1.036	1.036
E	0.818	0.938	1.005	1.005	1.013	1.013	1.021	1.021	1.029	1.029	1.037	1.037
F	1.368	1.361	1.006	1.006	1.014	1.014	1.022	1.022	1.030	1.030	1.038	1.038
G	1.609	1.608	1.007	1.007	1.015	1.015	1.023	1.023	1.031	1.031	1.039	1.039
H	1.605	1.547	1.008	1.008	1.016	1.016	1.024	1.024	1.032	1.032	1.040	1.040

0.000 ◄ Blank average

III - Enter standards concentration below in the ng/ml column
Note: To include S0 in the plot enter an arbitrary small number (e.g. 0.01) but not ZERO in C69
Note: To extrapolate the "fit" red curve add more concentrations immediately above (C61 and C60) and below Std data points (C68).

	ng/ml	signal	FIT	stdev
			1.752	
			1.752	
Enter S1	10.000	0.226	0.242	0.0718
Enter S2	2.500	0.318	0.308	0.0393
Enter S3	0.625	0.501	0.492	0.0355
Enter S4	0.156	0.878	0.899	0.0848
Enter S5	0.039	1.379	1.361	0.0263
Enter S6	0.010	1.609	1.621	0.0007
	0.001		1.735	
S0 (zero)		1.576	1.752	

4 - Adjust parameters a b c d (green cells) to optimize the fit.

1.609 1.000 0.159 0.226 ◄parameters first bet (please adjust green cells)

0.624 0.5014 IC50
0.876 3 0.159 0.8781 0.159
Calculations of initial curve parameters

1.752	0.936	0.123	0.217
(max) a	slope) b	IC50) c	(min) d

After you dump your data in the B46:M53 area
the first plot you'll see above will not be perfectly fitted to your data. You must adjust the parameters in the green cells above to optimize the fit of the red curve to your data.

0.0325 ◄This number gets smaller as the fit gets better

5 - Read unknown concentrations below. NOTE: trust results only if ODs are within the measuring range.
SPIKED (if you have "spiked" samples with known concentrations enter them in the F column)
▼ ▼ If the signal readings are as expected the result will be 100% X-reactivity

Smpls	duplic. 1	duplic. 2	Average	ng/ml	ng/ml	%Xreact
U1	1.001	1.001	1.001	0.117	10000	0%
U2	1.002	1.002	1.002	0.117	10000	0%
U3	1.003	1.003	1.003	0.117	10000	0%

Set up an 8 x 12 area to match the layout of the plate and copy the plate reader data in it.
Calculate the average of the blanks on another cell as indicated by the arrows starting from cells A1 and A2.
Enter the concentration of the standards (see under ng/ml in figure).
Calculate the average of the ODs of the standards and subtract the background (blank) as indicated by the arrows for the last standard (cells H1 and H2).
Plot a standard curve on a semi-log scale. Use the y axis for the average of the OD readings (minus the blank average) and the x axis for the standard concentrations in ng/ml.

Use the equation shown below to calculate the values on the "FIT" column and plot a smooth line of FIT values versus standard concentrations. Then change the parameters a (max), b (slope), c (IC_{50}), and d (min), until you are satisfied that fit is good.

$$y = \frac{\text{a - d}}{1 + (\text{x/c})^{\text{b}}} + d$$

Next calculate the average of your sample readings and subtract the blank average (see arrows starting from A3 and A4, and the arrows leading to "Average").
Finally, you may use the "reverse" of the equation above to calculate the concentrations in ng/ml for all your samples.

$$x = c\left(\frac{y-a}{d-y}\right)^{1/b}$$

Caution: when you calculate sample concentrations using the "reverse" equation if y = d or y > a or y < d, the reading is out of range and the calculation will yield an error or a meaningless negative concentration.

SUGGESTED PROTOCOL FOR SAMPLE EXTRACTION

If you are working with serum, you may spike it with known amounts of standard and check if they are accurately determined by the assay with and without extraction. Extraction eliminates potentially interfering substances, such as albumin. Extraction may also be necessary to concentrate the sample to within the measuring range. As with any purification technique, recovery of the desired substance is likely to be incomplete. Therefore, both optimization and quantification of the extraction procedure are recommended for more accurate determinations.

C18 Sep-Column Extraction Method

The following generic protocol is meant to help users with little experience in extracting their samples. It should be applicable to different biological fluids but should not be thought of as an optimized protocol for the kit antigen.

Required Materials

- SEP-COLUMN containing 200 mg of C18 (Cat. No. Y-1000)
- Buffer A (BUFF-A): 1% trifluoroacetic acid (TFA, HPLC Grade). (Acidifies plasma sample to remove interfering proteins such as albumin) (Cat. No. Y-1040)
- Buffer B (BUFF-B): 60% acetonitrile (HPLC Grade), 1% TFA, and 39% distilled water. (Elutes peptide from column) (Cat. No. Y-1045)
- You may also consider purchasing Extraction kits (Cat. No. S-5000), which include SEP-columns and buffers

Withdrawal and Preparation of Plasma

- Collect blood samples (2 - 6 ml) into a chilled syringe and transfer into a polypropylene tube containing EDTA (1 mg/ml of blood) as an anticoagulant and Aprotinin (500 KIU/ml of blood) as a protease inhibitor at 4°C. Do not use heparinized tubes as they may interfere with the assay. Vacutainers with EDTA are acceptable.
- Centrifuge blood at 1,600xg for 15 minutes at 4°C.
- Collect the top (plasma) layer.

- Proceed to extraction immediately or freeze at -70°C for later use.

Extraction Procedure

- Add an equal amount of Buffer A to the plasma.
- Centrifuge at 6,000xg to 17,000xg for 20 minutes at 4°C.
- Transfer supernatant to a new tube discarding any pellet that may be present.
- Equilibrate a SEP-COLUMN by washing with 1ml Buffer B followed by 3x 3ml Buffer A.
- Load the plasma solution onto the equilibrated SEP-Column.
- Slowly wash the column with Buffer A (3ml, twice) and discard the wash. A light vacuum (10 sec/drop) may be applied to the column.
- Elute the peptide slowly with Buffer B (3ml, once) and collect eluant in a polypropylene tube. A light vacuum may be applied as in previous step.
- Freeze-dry eluant to dryness using a dry ice/methanol bath to freeze the sample and a centrifugal concentrator to evaporate it
- Dissolve the residue in a suitable volume of ELISA buffer such that the concentration of the substance of interest will fall close to the IC_{50} (within the measuring range).

TROUBLESHOOTING

Most problems are caused by poor technique or by alterations of the protocol. Please check that the expiration date has not passed and store the kit properly and according to the storage section on page 2.

- ***Can the kit be used more than once?***
  - Although we do not guarantee the performance of our kits on a subsequent use, the end user should be able to use the kit multiple times if the reconstituted specific antiserum and Bt-tracer are stored at or below a constant -20 °C and the remaining of the components are kept dry and refrigerated (2-4 °C). Freezing aliquots of the reconstituted components may further extend multiple use lifetime.
- ***What are the sources of inaccurate readings?***
  - exceeding the OD range of plate reader
  - dirt or grease on the bottom of the plate - wipe with 70% ethanol
  - air bubbles or foaming in wells.
- ***The standard curve does not look right.***
  - If you wait too long to read, the curve will be flattened at the top. If you are not familiar with the kit we recommend you read the plate several times while the signal is still developing. For absorbance assays the developing blue color (absorb. 650 nm) will be less intense compared to that of the terminated reactions (yellow -absorb. 450 nm) but the data are still good and this way you won't risk losing the lower end of the range.
- ***The IC_{50} is not as expected***
  - Note that the IC_{50} reported with each of our products is based on the concentration of the prepared standards before they are added to the assay solution.
  - A difference by a factor of two or three may be normal for some kits and may be caused by the time it takes to equilibrate the binding of the tracer and the standard. This will be especially true for pre-incubation protocols. If possible you

should always include your own reliable standard at a concentration close to the expected IC_{50} to check the accuracy of the kit.
  - In cases where the standard curve is almost rectilinear, accurate IC_{50} values cannot be calculated.
  - Using excessive amounts of antiserum or tracer, or using a degraded standard may elevate the IC_{50}.
- ***There is too much variation in duplicated readings.***
  - There are only trivial explanations for this such as: (a) poor mixing, (b) poor pipetting technique or faulty pipettes, (c) kit reagents not allowed to equilibrate to room temperature before use, (d) cross-contamination of samples, e.g. droplets or spray from one well to the next, (e) bubbles or foaming in the wells, or finger prints or dirt on the bottom of the plate, etc.
- ***The readings are lower than expected.***
  - The color intensity has little to do with the accuracy of the kit, as long as the slope in the measuring range is normal, but, if the intensity is extremely low, and assuming that you have waited long enough, this may mean that one of the components (antiserum, Bt-tracer, SA-HRP, TMB substrate) was added in low amounts or was degraded due to incorrect storage or excessive freeze-thawing.
- ***The curve looks OK but the results seem implausible.***
  - Possibly you used different solvents or conditions for standards and samples.
  - The antiserum may bind to another antigenically similar peptide
  - Antigen was lost during extraction or extraction did not eliminate interfering factors
  - Make sure that the kit's antigen is the same as the target that you are trying to measure. Sometimes the kit's antigen is a peptide that is part of but not the complete natural protein. If so the kit can still be used for determining relative concentrations but not necessarily for determining the absolute concentration of the complete protein antigen.

REFERENCES

1) **T. Porstmann and S.T. Kiessig** Enzyme immunoassay techniques. An Overview. J. Immunol. Methods **150**, 5-21 (1992)
2) **S. Avrameas** Amplification systems in immunoenzymatic techniques. J. Immunol. Methods **150**, 23-32 (1992)
3) **E. Bucht et al.** A rapid extraction method for serum calcitonin. Clin. Chim. Acta, **195**, 115-124 (1991)

SAFETY PRECAUTIONS

The physical and chemical properties of the reagents contained in this kit have been tested individually. Reagents do not contain ingredients which have been determined to be health hazards and which comprise greater than 1% of the mixture or which could be released from the mixture in concentrations that would exceed OSHA permissible exposure limits.

Hazardous Ingredients

The lyophilized antiserum and biotinylated tracer contain ProClin. The ELISA buffer concentrate contains Tris and ProClin. The buffer is in liquid form. The SA-HRP contains 0.01% methylisothiazolone, 0.01% bromonitrodioxane, and 10 ppm Proclin 300 as a preservative.

Physical and Chemical Data

Components are stable in closed containers under normal temperatures and pressures. No hazardous polymerization is known.

Fire and Explosion Data

Components are non-combustible with negligible fire hazard when exposed to heat or flame. Fire fighting media should be appropriate to burning material.

Health Hazards

Components may be harmful by inhalation, ingestion, or skin absorption and may cause skin irritation or eye irritation. In case of eye contact, flush eye with water and contact a physician. In case of skin contact, wash skin with soap and water.

Reactivity Data

Components are stable in closed containers under normal temperatures and pressures.

Spill and Disposal Procedures

For spills, ventilate area and wash spill site. For disposal, please dispose in accordance with local regulations.

Handling and Storage Information

Safety glasses, gloves, and a full-length lab coat should be worn to prevent unnecessary contact.

The above information is believed to be correct but does not purport to be all-inclusive and shall be used only as a guide. It is the user's responsibility to determine the suitability of this information for the adoption of safety precautions as may be necessary. Peninsula Laboratories International, Inc. shall not be held liable for any damage resulting from the handling or use of the above product.

GUARANTEE AND LIMITATION OF REMEDY

Peninsula Laboratories International, Inc. makes no guarantee of any kind, expressed or implied, which extends beyond the description of materials in this kit, except that these materials and kit will meet our specifications at the time of delivery. The customer's remedy and Peninsula Laboratories International, Inc.'s sole liability hereunder are limited at Peninsula Laboratories International, Inc.'s option to refund the purchase price or replace material that does not meet our specifications. By the acceptance of our products, the customer indemnifies and holds Peninsula Laboratories International, Inc. harmless against and assumes all liability for the consequences of its use or misuse by the customer, its employees or others.

__/__/____ S-_____ Lot _____ _____
_____
_____

12								
11								
10								
9								
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